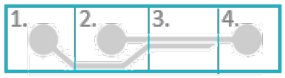
1. Objective

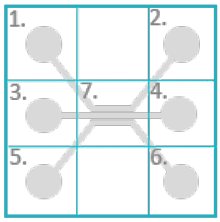
This protocol describes the procedure for immunostaining of tissues grown in the OrganoPlate® 2-lane and 3-lane (see chip layout below)

OrganoPlate® 2-lane



1. Gel inlet
2. Medium inlet
3. Observation window
4. Medium outlet

OrganoPlate® 3-lane



1. Top medium inlet
2. Top medium outlet
3. Gel inlet
4. Gel outlet
5. Bottom medium inlet
6. Bottom medium outlet
7. Observation window

2. Background

Immunofluorescent staining is a technique that uses antibodies to target specific cellular biomolecules expressed on cells. Detection of the bound antibodies is done using fluorescence microscopy.

3. Materials

- Tween-20 (Sigma, cat# P9616)
- FBS (Gibco/ATCC, cat# A13450)
- Triton™ X-100 (Sigma, cat# T8787)
- HBSS (+Ca/Mg) (Sigma, cat# 55037C-1000ML)
- BSA (Sigma, cat# A2153)
- Crushed ice
- Repeating multichannel pipets and tips
- 1x PBS (Gibco, cat# 70013065)
- Rocker platform (optional)
- Fixative (see table 1)
    - Standard fixative is 3.7% formaldehyde (diluted 1:10 from stock, Sigma, cat# 252549-1L)

Fixative	Incubation time
3.7% formaldehyde in HBSS	10-15min RT
0.4% formaldehyde in HBSS	10-15min RT
-20°C 100% acetone	5min RT
-20°C 100% methanol	10-15min RT
-20°C 95% methanol, 5% acetic acid	5-10min RT

Table 1. Fixation methods compatible with the OrganoPlate®

- Permeabilization buffer: 0.3% Triton X-100
- Blocking solution: 2% FBS, 2% BSA, 0.1% Tween20 in PBS
- Washing solution: 4% FBS in PBS
- Antibodies and nuclear stain (Hoechst™/DraQ5™)

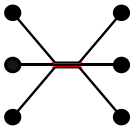
4. Assay

Fixation

All steps are performed at room temperature

1. Prepare fixative (see table 1)
    a. Standard fixative is 3.7% formaldehyde in HBSS (dilute 1:10 from 37% stock)
    b. Complete OrganoPlate® 2-lane: 21 mL
    c. Complete OrganoPlate® 3-lane: 17 mL
2. Aspirate medium from the chips and add **fixative** to the chips' inlets and outlets according to the volume scheme below
    a. OrganoPlate® 2-lane: 50 µL to gel inlet, 100 µL to medium inlet, 50 µL to medium outlet
    b. OrganoPlate® 3-lane: 100 µL to top medium inlet, 50 µL to all other inlets and outlets

50µL	**100**µL		50µL



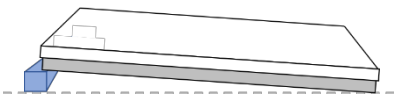
100µL		50µL
50µL		50µL
50µL		50µL

3. Incubate the fixative for the appropriate amount of time (see table 1)
4. Aspirate fixative and wash chips 2x (5 min each) with **PBS** using the volume scheme shown in step 2
5. Proceed to immunostaining or seal the plate around the edges with Parafilm® and wrap the plate in aluminum foil. The plate can be stored at RT up to 2 weeks.

Immunostaining

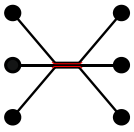
All steps are performed at room temperature unless specified otherwise

To allow successful binding of primary and secondary antibodies, antibody is perfused through the OrganoPlate® during antibody incubation steps. Perfusion can be created by placing the OrganoPlate® on a regular rocker platform and having it switch sides. Use a small angle and a low switching interval (i.e. 5° angle, 2-5 min interval). Alternatively, flow can be induced by placing the OrganoPlate® under an angle by positioning one end on top of an object (see figure on the right) and regularly switching sides.



6. Prepare permeabilization buffer, blocking solution, and washing solution (see materials list)
7. Wash chips 1x 5 min with **washing solution** according to the volume scheme below
    a. OrganoPlate® 2-lane: 50 µL to gel inlet, 100 µL to medium inlet, 50 µL to medium outlet
    b. OrganoPlate® 3-lane: 100 µL to top medium inlet, 50 µL to all other inlets and outlets

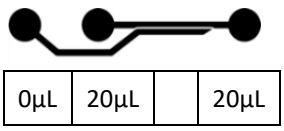
50µL	**100**µL		50µL



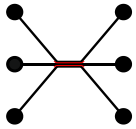
100µL		50µL
50µL		50µL
50µL		50µL

8. **Permeabilize** cells for 10 minutes with permeabilization buffer according to the volume scheme shown in step 7

9. Wash chips 1x 5 min with **washing solution** using the volume scheme shown in step 7
10. Block cells for 30-45 min with **blocking solution** according to the volume scheme shown in step 7
11. Meanwhile, prepare primary antibody in blocking solution in the appropriate dilutions
    a. OrganoPlate® 2-lane: 40 µL antibody per chip
    b. OrganoPlate® 3-lane: 80 µL antibody per chip
12. Aspirate blocking solution and add **primary antibody solution** according to the volume scheme below

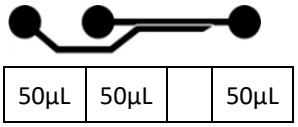


0µL	20µL		20µL

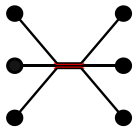


25µL		25µL
0µL		0µL
15µL		15µL

13. Incubate primary antibody on the rocker platform at RT (or at 4°C if desired)
    a. For tubular cultures, 1-2 hours of incubation on the rocker platform at RT is sufficient
    b. For cultures of cells in gel, longer incubation times may be required (see page 4)
14. Meanwhile, prepare secondary antibody in blocking solution in the appropriate dilutions
    a. OrganoPlate® 2-lane: 40 µL antibody per chip
    b. OrganoPlate® 3-lane: 80 µL antibody per chip
15. Wash chips 2x (3 min each) with **washing solution** using the volume scheme shown in step 7
16. Add **secondary antibody solution** according to the volume scheme shown in step 12
17. Incubate secondary antibody in the dark on the rocker platform at RT
    a. For tubular cultures, 30 minutes of incubation on the rocker platform at RT is sufficient
    b. For cultures of cells in gel, longer incubation times may be required (see page 4)
18. Wash chips 2x (3 min each) with **washing solution** using the volume scheme described in step 7
19. If desired, stain cells with direct stains (e.g. Hoechst or ActinRed), using manufacturer's instructions
    a. Use stains for fixed cells
    b. Use the volume scheme shown in step 12
    c. Incubate stains on the rocker at RT at least 15 min for cells grown as tubes against the ECM gel and at least 30 min for cells embedded in ECM gel (see page 4)
20. Wash chips 1x (5 min) with **PBS** according to the volume scheme shown in step 7
21. Aspirate all wells and add 50 µL of **PBS** to all wells



50µL	50µL		50µL



50µL		50µL
50µL		50µL
50µL		50µL

22. Proceed to microscopy or store the plate
    a. Perform microscopy within one week after staining for optimal results
    b. Imaging can be performed on all standard fluorescent microscopes
    c. Store plate by sealing edges with Parafilm® and wrapping it in aluminum foil. Store at RT for up to two weeks

5. Troubleshooting

Insufficient staining

Depending on the setup of the culture, some cultures may require longer staining procedures to obtain optimal results, for example neuronal networks grown in Matrigel® in the OrganoPlate® 2-lane. For these types of cultures, the following measures can be taken to improve antibody staining:

- Addition of 1% Triton to the primary and secondary antibody solution (steps 11 & 14)
- Prolongation of incubation times
    - Incubate primary antibody for 2 days, on the rocker, at RT (step 13b)
    - Incubate secondary antibody for 2 days, on the rocker, at RT (step 17b)
    - Incubate nuclear stains for 1 day, on the rocker, at RT (step 19c)

Note: because these measures prolong the staining procedure significantly, use of aseptic technique is recommended to avoid growth of bacteria in the cultures. After fixation, work in a sterile cabinet and use sterile solutions (prepare solutions using only sterile components or filter buffers after preparation).

MIMETAS product list

Cat. No.	Product Name
MI-AR-CC-01	OrganoReady® Caco-2
9605-400-B	OrganoPlate® 2-lane
4004-400-B	OrganoPlate® 3-lane 40
6405-400-B	OrganoPlate® 3-lane 64
6401-400-B	OrganoPlate® Graft
MI-OFPR-S	OrganoFlow® S
MI-OFPR-L	OrganoFlow® L
MI-OT-1	OrganoTEER®

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